PROJECT REPORT
ARIZONA ICEBERG LETTUCE RESEARCH COUNCIL

For Period: July 1, 2006-June 30, 2007

Project Title: Determination of *Mirafiori Lettuce Virus* and *Lettuce Big Vein Virus* Incidence and Genetic Variability in Big Vein Infected Lettuce

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INTRODUCTION:

Lettuce big vein, a viral disease vectored by the soil-borne fungus *Olpidium brassicae*, was first identified by Jagger and Chandler (1934), and is a serious disease throughout all western lettuce production regions. Symptoms include chlorosis surrounding the vascular bundles in the leaf and increased stiffness of the leaves, giving a bushy appearance and preventing head formation. Big vein is most prevalent in cool wet soils (Campbell and Grogan 1963, Westerlund *et al.* 1978a, 1978b). The disease increases in frequency with continual lettuce production without rotation. Effective long-term control of big vein disease can only be accomplished through genetic resistance, and is an important component to sustaining quality lettuce production in California.

Although big vein disease has impacted lettuce production for many years, the causal agent was only recently identified. As a result, disease control measures have been slow to develop. Recent work in Europe identified a new virus, *Mirafiori lettuce big vein virus* (MLBVV) as the causal agent for big vein disease (Lot et al., 2002). This virus was known only as *Mirafiori lettuce virus* (MiLV) until recent taxonomic changes were issued. Another virus, *Lettuce Big Vein associated Virus* (LBVaV), was previously found associated with big vein disease, but a causative relationship was never confirmed (LBVaV was formerly known as *Lettuce big vein virus* [LBVV]). The European studies demonstrated that plants exhibiting big vein symptoms were always infected with MLBVV, but were often coinfected with LBVaV as well. All evidence to date suggests this is merely an association based on a common vector, and that LBVaV does not contribute to disease. Knowledge of the virus responsible for big vein disease provides an opportunity to develop more effective methods of screening for resistance by identifying plants with reduced virus accumulation, as well as reduced symptom production. Coupling these methods will facilitate more reliable resistance testing than those used previously by lettuce breeders.

It is currently unknown how big vein resistance relates to accumulation of MLBVV, outside of the simple fact that MLBVV accumulation is one requirement for symptom expression. Understanding the genetic variation for maximum allowable MLBVV accumulation, and the genetic variation for the MLBVV concentration required for symptom expression is crucial for lettuce breeders. Multiple sources of resistance exist, and it is not clear which sources of

resistance will result in the highest level of resistance. For example, current partially resistant *L. sativa* cultivars, such as Pavane and Pacific, exhibit reduced frequency of symptomatic plants. In contrast, some *L. virosa* accessions have no symptom development (Hayes et al., 2006), but may or may not accumulate MLBVV to detectable levels (unpublished). Efforts to introgress resistance from *L. virosa* into lettuce using accession IVT280 was reported this spring (Hayes and Ryder, 2007). Hybrid breeding populations did not contain individuals with complete resistance, but did have variation for a high level of partial resistance that was likely based on novel alleles derived from *L. virosa*. This suggests that the mechanism of resistance in *L. virosa* is different from the mechanism conferring partial resistance in cultivated lettuce. While these results highlight the complexities of this material, they have also instigated several private seed companies to initiate big vein resistance breeding efforts with *L. virosa*. Futher research to understand the relationships between MLBVV accumulation and symptom expression should result in more effective sources of resistance. Furthermore, excessive reliance on a single form of resistance can lead to pathogen evolution that ultimately overcomes resistance. By combining or alternating big vein resistance sources, we intend to improve long-term control of this economically damaging disease. This information will allow breeding programs to focus not only on reducing symptom development, but also virus incidence, leading to more rapid development and more reliable resistance against big vein disease than is possible using current methods.

PREVIOUS ACCOMPLISHMENTS:

- Confirmed both MLBVV and LBVaV are associated with big vein disease in western US lettuce production regions, and confirmed the universal presence of MLBVV in big vein symptomatic tissue.

- Demonstrated that American MLBVV and LBVaV isolates are genetically very closely related to isolates of these viruses from other parts of the world.

- Developed RT-PCR primer sets and nucleic acid spot hybridization for sensitive, rapid and efficient detection of MLBVV and LBVaV.

- Developed primers for measurement of MLBVV accumulation in lettuce by quantitative RT-PCR.

- Determined that although IVT280 is highly resistant to MLBVV and LBVaV, MLBVV can replicate to some degree under some conditions in IVT280 and other *L. virosa* accessions.

LONG RANGE OBJECTIVE:

Given the recent advances that identified MLBVV as the pathogen responsible for v big vein disease, and our recent development of tools for the efficient detection and monitoring of MLBVV in lettuce, research into MLBVV resistance will likely to lead to important breakthroughs and improvements in big vein resistance. The long range objectives of this

research are to identify diverse sources of big vein resistance that reduce MLBVV accumulation and have 0% symptomatic plants, and to develop tools to introgress this resistance into adapted cultivars. Originally we had anticipated 2006-2007 would be the last year for this project, however due to the complexities of MLBVV accumulation patterns in *L. virosa* and *L. virosa* hybrids that have resulted in the need for more careful analysis and the need to consider less costly analysis methodologies it will be necessary to continue these experiments into the next AILRC budget year in order to complete this research.

OBJECTIVES FOR 2006-2007:

1. Determine LBVaV and MLBVV accumulation in BC_1F_3 progeny of *L. sativa x L.* hybrids, *L. virosa* accessions, tolerant *L. sativa* cultivars, and susceptible *L. sativa* cultivars using Real-Time RT-PCR.

2. Test *L. virosa x L. sativa* F_1 hybrids for infection by both MLBVV and LBVaV using traditional RT-PCR to determine whether apparent complete resistance (lack of virus accumulation) in *L. virosa* is dominant to susceptibility in *L. sativa*.

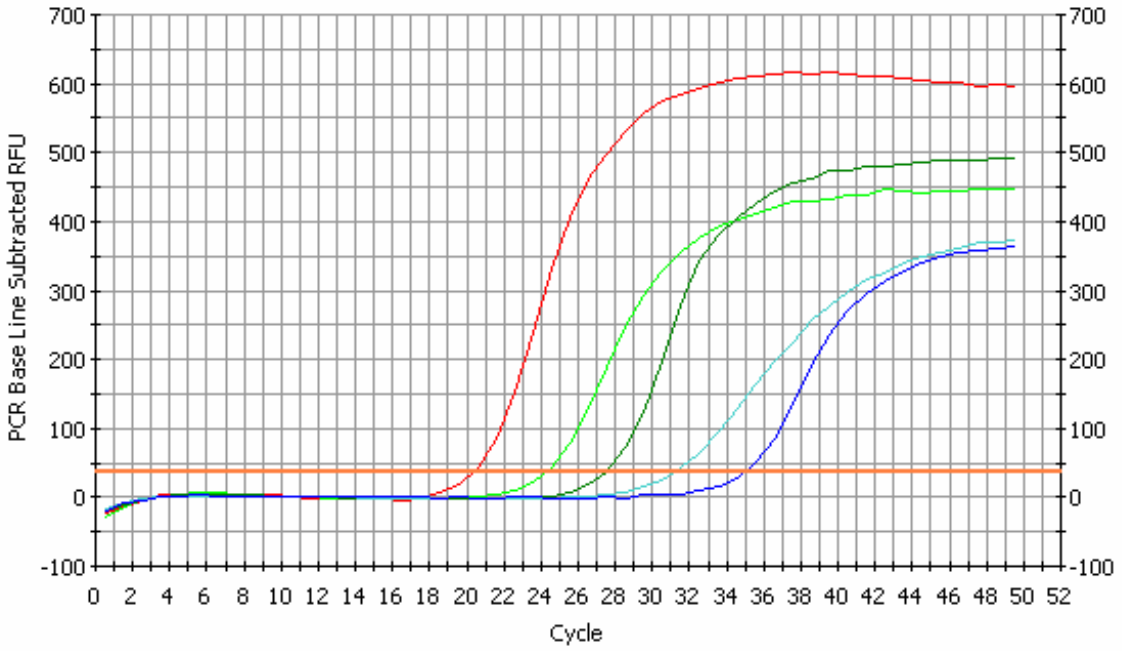
METHODS AND RESULTS:

OBJECTIVE 1: Determine LBVaV and MLBVV accumulation in BC_1F_3 progeny of *L. sativa x L.* hybrids, *L. virosa* accessions, tolerant *L. sativa* cultivars, and susceptible *L. sativa* cultivars using Real-Time RT-PCR.

Development and testing of real-time RT-PCR for determination of MLBVV concentration in lettuce (main objective).

Quantitative RT-PCR (often referred to as "real-time" RT-PCR) for determination of the amount of virus present in an infected plant, should focus on genes that are not expressed in massive numbers in order to more accurately reflect the true amount of virus present. In other words, it is better to focus on a non-structural gene, such as one involved with virus replication, rather than on one that is produced in excess, such as the RNA encoding the coat protein, for which many copies are produced on shorter, "subgenomic" RNAs. This more accurately reflects the amount of virus in a given plant than does use of a high copy viral RNA. With this in mind, we designed DNA primers for quantitative RT-PCR (qRT-PCR) to sections of the polymerase region of MLBVV, encoded on MLBVV RNA1 (There are 4 different RNAs that make up the genome of MLBVV). The primers should be effective against most MLBVV isolates from throughout the world, based on sequence conservation among isolates as documented in our previous research (Hayes et al., 2006) and that of others. Initially we began by testing our traditional RT-PCR primers against MLBVV RNA 1 sequences, and this was initially described in last year's report. These primers were fairly effective, but were still giving some small degree of misamplification, which could interfere with reliable quantification of virus concentrations. As a result, new primers were designed to amplify another portion of MLBVV RNA1. These new primers accurately detect MLBVV alone, without misamplification, and can quantitatively determine levels of MLBVV in infected lettuce tissue. An example of such an amplification is shown in Fig. 1.

Fig. 1. Graph of fluorescence vs. RT-PCR cycle (for five 10-fold dilutions of a sample containing MLBVV), performed with SYBR Green fluorescent dye. Fluorescence correlates with amount of RT-PCR product.



The difficulty with quantitative RT-PCR is the cost associated with doing large numbers of samples. While the approach is quite effective, it is not feasible for analyzing large numbers of samples on a limited budget. Consequently we chose to explore alternatives that may not be quite as accurate in determining virus concentration as qRT-PCR, but still may be equally effective in differentiating lines with high or intermediate levels of MLBVV accumulation from those with low levels of MLBVV accumulation or none at all.

SUB-OBJECTIVE 1A: Compare real-time RT-PCR detection with serological detection using antiserum against MLBVV.

During the course of our studies we determined that a German company markets an antiserum that is supposed to detect MLBVV. We purchased this antiserum and tested it using both ELISA (Enzyme-linked immunosorbent assay) and immunoblot procedures, hoping that this would be an inexpensive, yet effective method for quantifying virus levels in *L. virosa* and *L. sativa* breeding lines.

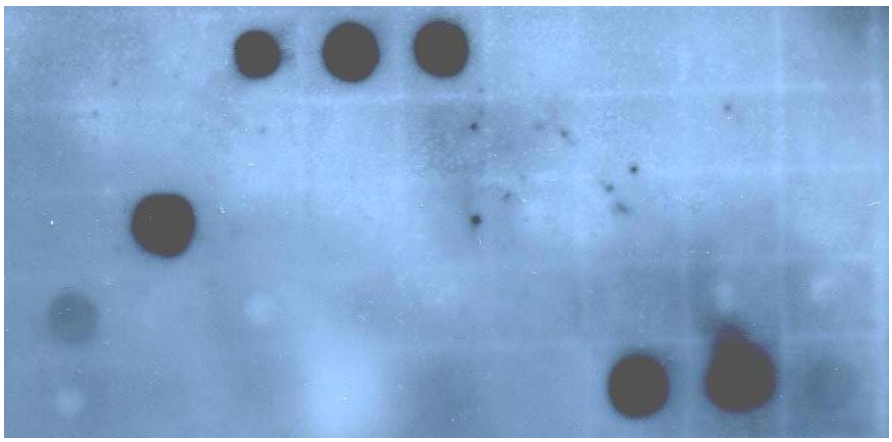
We obtained field samples of lettuce (*L. sativa*) and *L. virosa* exhibiting classic big vein symptoms, greenhouse-grown samples in which MLBVV was introduced into soil to infect lettuce plants, healthy lettuce grown in soil free of MLBVV and its vector, *O. brassicae*, and samples confirmed by RT-PCR to be infected with or be free of MLBVV. These samples were tested in separate experiments using ELISA or immunoblotting, following standard

procedures. Both methods resulted in an inability to distinguish between known positives and negatives (data not shown). This suggested that the antiserum was actually cross-reacting with healthy *Lactuca sativa* or *L. virosa*. In other words, the antiserum will NOT reliably identify the presence or absence of MLBVV and will NOT be useful for breeding programs or laboratory diagnostics based on our results. It will be much more reliable to depend on our RT-PCR, quantitative RT-PCR or other diagnostic technologies.

SUBOBJECTIVE 1B: Determine if hybridization testing comparing relative MLBVV levels can effectively differentiate breeding lines for level of virus accumulation.

Since serological detection of MLBVV is not an option, we also explored nucleic acid spot hybridization (or dot blot hybridization) as a lower-cost alternative to quantitative RT-PCR. We developed this method in the summer of 2005 and now revisit it as an economical method for quantifying the amount of MLBVV present in *Lactuca* germplasm. Although this method is not as accurate for determining virus concentration as quantitative RT-PCR, it can effectively differentiate plants with no virus accumulation or very low levels of accumulation from those with moderate or high levels. This should provide the answers necessary for germplasm analysis and will result in a project that may provide results more rapidly and with greatly reduced cost than would be possible using the quantitative RT-PCR (real-time) method. An example of one of these tests is illustrated in Fig. 2, below.

Figure 2: Differential detection of *L. virosa* with (dark or light spots) and without (no spots) MLBVV infection as confirmed by dot blot hybridization with a MLBVV-specific probe.



OBJECTIVE 2: Test *L. virosa x L. sativa* F_1 hybrids for infection by both MLBVV and LBVaV using traditional RT-PCR to determine whether apparent complete resistance (lack of virus accumulation) in *L. virosa* is dominant to susceptibility in *L. sativa*.

Using the hybridization method described above, we screened 245 samples of 7 different *L. virosa* lines, along with *L. sativa* controls, from a greenhouse resistance test to determine

MLBVV accumulation in each. Results (shown in Fig. 3) clearly illustrate the value of this hybridization method for differentiating the performance of breeding lines, showing variation for virus accumulation over two sampling dates, and greater sensitivity than is possible from relying on symptoms alone. Additional studies will continue with this material.

Fig. 3. Variation in symptoms and MLBVV accumulation among L. virosa breeding lines

Line	No Tested	% symptomatic	% MLBVV Positive
Reading date 1: 120 days after planting			
CGN16275	**11**	**45b**	**73b**
CGN16276	**26**	**0a**	**8a**
CGN16277	**11**	**27b**	**55b**
IVT280	**58**	**0a**	**24a**
PI274378	**59**	**0a**	**20a**
SAL012	**60**	**0a**	**8a**
Reading date 2: 180 days after planting			
CGN16275	**10**	**82b**	**80c**
CGN16276	**26**	**31a**	**77c**
CGN16277	**11**	**82b**	**91c**
IVT280	**50**	**0a**	**51bc**
PI274378	**50**	**0a**	**8ab**
SAL012	**56**	**0a**	**0a**

Publications directly resulting from this project to date:

Hayes, R.J., Wintermantel, W.M., Nicely, P.A., and Ryder, E.J. 2006. Host resistance to Mirafiori lettuce big-vein virus and Lettuce big-vein associated virus and virus sequence diversity and frequency in California. Plant Disease 90: 233-239.

Hayes, R.J. and E.J. Ryder EJ. 2007. Introgression of novel alleles for partial resistance to big vein disease from *Lactuca virosa* into cultivated lettuce. Hortscience 42:35-39.

Hayes, R.J., Ryder, E.J., and Wintermantel, W.M. 2007. Genetic variation for big vein disease symptom expression and *Mirafiori lettuce big vein virus* incidence in *Lactuca virosa* L., a wild relative of cultivated lettuce. (in preparation).